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Protein Therapeutics

Intracellular delivery of protein and peptide therapeutics

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Many proteins and peptides are used as highly specific and effective therapeutic agents. Their use is, however, complicated by their instability and side effects. Because many protein and peptide drugs have their therapeutic targets inside cells, there is also an important task to bring these drugs into target cells without subjecting them to the lysosomal degradation. This review describes current approaches to the intracellular delivery of protein and peptide drugs. Various drug delivery systems and methods are considered allowing for safe and effective transport of protein and peptide drugs into the cell cytoplasm.

Peptide and protein drugs – brief overview and delivery problems

Many proteins and peptides possess biological activity that makes them potent therapeutics. Enzymes represent an important and, probably, the best investigated group of protein drugs. Their clinical use has already a rather long history [1–3]. Certain diseases (usually inherited) connected with the deficiency of some lysosomal enzymes (so-called storage diseases) can be treated only by the administration of exogenous enzymes [4,5]. In general, therapeutic enzymes include: antitumor enzymes acting by destroying certain amino acids required for tumor growth; enzymes for replacement therapy (usually digestive enzymes) for the correction of various insufficiencies of the digestive tract; enzymes for the treatment of lysosomal storage diseases; enzymes for thrombolytic therapy; antibacterial and antiviral enzymes; and hydrolytic and anti-inflammatory enzymes.

Peptide hormones, first of all insulin, are among the most broadly used drugs. More recently, peptides such as somatostatin analogs (octreotide, lanreotide, vapreotide) become available in the clinic for the treatment of pituitary and gastrointestinal tumors [6]. Peptide inhibitors of angiogenesis including endostatin are currently in different stages of clinical trials and show a great promise for cancer treatment [7,8]. Research on depsipeptides has also revealed a set of potential anticancer agents [9]. Antibodies against certain cancer-specific ligands can also be considered as protein anticancer drugs [10–12].

Still, the use of proteins and peptides as therapeutic agents is hampered by the whole set of their intrinsic properties associated with their nature as complex macromolecules, which are, as a rule, foreign to the recipient organism. This leads to low stability of the majority of peptide and especially protein drugs at physiological pH values and temperatures, particularly when these proteins have to be active in the environment different from their normal one. Different processes leading to the inactivation of various biologically active proteins and peptides *in vivo* include: conformational protein transformation into inactive form due to the effect of temperature, pH, high salt concentration or detergents; the dissociation of subunit proteins into the individual subunits

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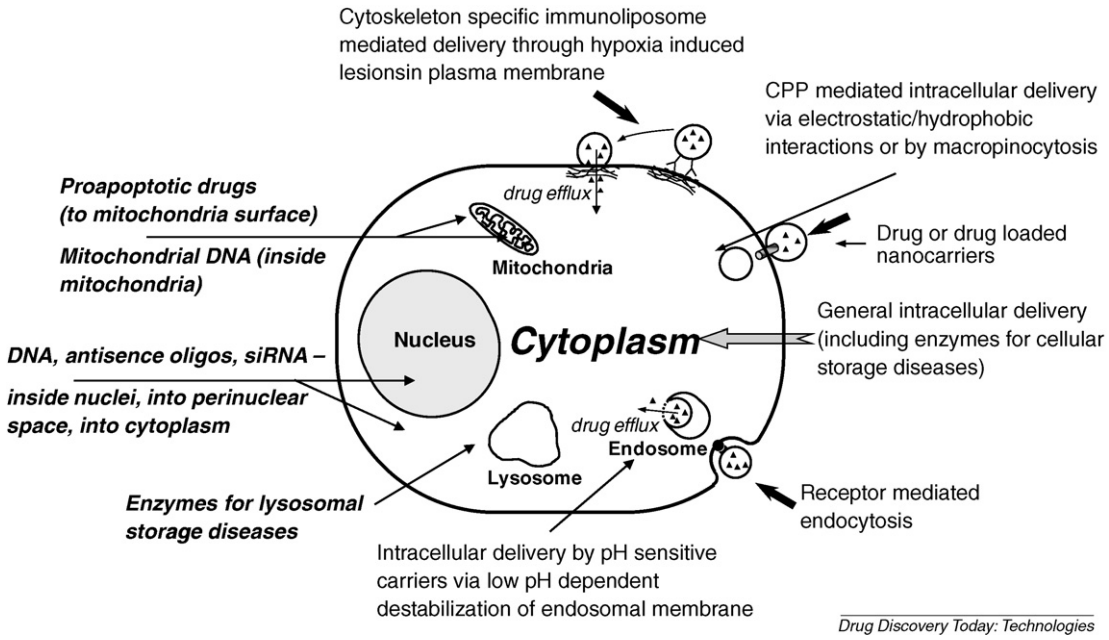

Figure 1. General scheme of intracellular drug delivery. Types of diseases, organelles involved and required drugs are shown together with possible delivery routes and protocols.

or in case of cofactor-dependent enzymes, enzyme-cofactor complexes and the association of protein or peptide molecules with the formation of inactive associates; non-covalent complexation with ions or low-molecular-weight and high-molecular-weight compounds, affecting the native structure of the protein or peptide; proteolytic degradation under the action of endogenous proteases; chemical modification by different compounds in solution (for example, oxidation of SH-groups in sulfhydryl-containing enzymes and Fe (II) atoms in heme-containing proteins by oxygen; thiol-disulfide exchange, destruction of labile side-groups like tryptophan and metionine). All these lead to rapid inactivation and rapid elimination of exogenous proteins from the circulation mostly because of renal filtration, enzymatic degradation, uptake by the reticuloendothelial system (RES) and accumulation in non-targeted organs and tissues. Rapid elimination and widespread distribution into non-targeted organs and tissues requires the administration of a drug in large quantities, which is often not economical and sometimes complicated owing to non-specific toxicity. A very important point is also the immune response of the macroorganism to foreign proteins containing different antigenic determinants. There exist also certain problems associated with the biological mechanisms of drug action. Many peptide and protein drugs as well as antibodies exert their action extracellularly, by receptor interaction. Many others, however, have their targets inside the cell. In the latter case, low permeability of cell membranes to macromolecules often represents an additional obstacle for the development of peptide-based and protein-based drug formulations.

Intracellular targets and intracellular drug delivery – pharmaceutical carriers

Many pharmaceutical agents, including various large molecules (proteins, enzymes, antibodies) and even drug-loaded pharmaceutical nanocarriers, need to be delivered intracellularly to exert their therapeutic action inside cytoplasm or onto nucleus or other specific organelles, such as lysosomes, mitochondria or endoplasmic reticulum (Fig. 1). Intracellular transport of different biologically active molecules is one of the key problems in drug delivery in general. In addition, the introcytoplasmic drug delivery in cancer treatment might overcome such important obstacle in anticancer chemotherapy as multidrug resistance. However, the lipophilic nature of the biological membranes restricts the direct intracellular delivery of such compounds. The cell membrane prevents big molecules such as peptides, proteins and DNA from spontaneously entering cells unless there is an active transport mechanism as in case of some short peptides. Under certain circumstances, these molecules or even small particles can be taken from the extracellular space into cells by the receptor-mediated endocytosis [13]. The problem, however, is that every molecule/particle entering cell via the endocytic pathway becomes entrapped into endosome and eventually ends in lysosome, where active degradation processes under the action of the lysosomal enzymes take place. As a result, only a small fraction of unaffected substance appears in the cell cytoplasm. As a result, many compounds showing a promising potential *in vitro*, cannot be applied *in vivo* owing to bioavailability problems. So far, multiple and only partially successful attempts have been made to bring various

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macromolecular (protein/peptide) drugs and drug-loaded pharmaceutical carriers directly into the cell cytoplasm bypassing the endocytic pathway, to protect drugs from the lysosomal degradation, thus enhancing their efficiency. The methods like microinjection or electroporation used for the delivery of membrane-impermeable molecules in cell experiments are invasive in nature and could damage cellular membrane [14,15]. Much more efficient are the non-invasive methods, such as the use of pH-sensitive carriers including pH-sensitive liposomes [16] and cell-penetrating molecules (see further).

In many cases, to increase the stability of administered drugs, to improve their efficacy and decrease undesired side-effects and even to assist in better intracellular delivery various pharmaceutical carriers are used. One can name liposomes among the most popular and well-investigated drug carriers. Liposomes are artificial phospholipid vesicles with the size varying from 50 to 1000 nm, which can be loaded with a variety of drugs. Liposomes are considered as promising drug carriers for well over two decades [17,18]. They are biologically inert and completely biocompatible; they cause practically no toxic or antigenic reactions; drugs included into liposomes are protected from the destructive action of the external media. Association of drugs with carriers, such as liposomes, results in delayed drug absorption, restricted drug biodistribution, decreased volume of drug biodistribution, delayed drug clearance and retarded drug metabolism [19]. Plain liposomes are rapidly eliminated from the blood and captured by the cells of the reticulo-endothelial system (RES), primarily, in liver and spleen, as the result of rapid opsonization of the liposomes. Most liposomes are internalized by phagocytic cells via endocytosis and destined to lysosomes for degradation [20]. The use of targeted liposomes, that is, liposomes selectively accumulating inside the affected organ or tissue, might increase the efficacy of the liposomal drug and decrease the loss of liposomes and their contents in RES. To obtain targeted liposomes, many protocols have been developed to bind corresponding targeting moieties including antibodies to the liposome surface [17,21]. However, the majority of antibody-modified liposomes still accumulate in the liver, which hinders their significant accumulation in target tissues, particularly those with a diminished blood supply (ischemic or necrotic areas) and/or those with a low concentration of a target antigen. Dramatically better accumulation can be achieved if the circulation time of liposomes could be extended leading to the increased total quantity of immunoliposomes passing through the target and increasing their interactions with target antigens. This is why long-circulated (usually, coated with polyethylene glycol, PEG, that is, PEGylated) liposomes have attracted so much attention over the past decade [22]. It was also demonstrated [23] that unique properties of long-circulating and targeted liposomes could be combined in one preparation, where antibodies or other specific binding molecules have been attached to the water-exposed tips of PEG chains [24].

Liposomes as pharmaceutical carriers for protein and peptide drugs – pH-sensitive liposomes for cytoplasmic drug delivery

Peptides and proteins in liposomes

One of the most popular and well-elaborated technologies to improve pharmacological properties of proteins and peptides is their incorporation into liposomes, and liposomal forms of various enzymes have been prepared and investigated: glucose oxidase, glucose-6-phosphate dehydrogenase, hexokinase, β-galactosidase, β-glucuronidase, glucocerebrosidase, α-mannosidase, amiloglucosidase, hexoseaminidase A, peroxidase, β-D-fructofuranosidase, neuraminidase, superoxide dismutase and catalase, asparaginase, cytochrome oxidase, ATPase, dextranase and many other enzymes from different sources (see [25] for review).

From the clinical point of view, the potential ability of liposome-encapsulated enzymes to enter the cytoplasm or lysosomes of live cells is of primary importance for the treatment of inherited diseases caused by the abnormal functioning of some intracellular enzymes, especially in liver and CNS cells [5,26]. The use of liposome-immobilized enzymes instead of their native precursors opens new opportunities for enzyme therapy [27,28] especially in the treatment of diseases localized in liver cells that are natural targets for liposomes. Thus, the biodistribution of liposomes containing β-fructofuranosidase has been studied [29]. It was shown that within an hour, 50% of the administered enzyme can still be found in the circulation, and the enzyme preserves its activity for a long time – 25% of the administered activity can be found in the liver after 48 h. β-Glucuronidase, immobilized into charged liposomes composed mainly from phosphatidyl choline dipalmitoyl, also demonstrated fast accumulation in the liver of experimental mice. The enzyme remained active more than a week, associated with the lysosomes of liver cells [30]. The ability of liposome-immobilized β-galaclosidase to degrade GM_1-ganglioside in lysosomes of feline fibroblasts with pathological accumulation of this substrate has been demonstrated [31]. The incorporation of liposome-encapsulated therapeutic enzymes into appropriate cells could be enhanced by rather simple methods [32]. Thus, β-glucocerebrosidase included into liposomes was predominantly captured by Kupffer cells in the liver; the modification of liposomes with mannoside residues increased the capture because of the presence of mannose-specific receptors on target cells. β-Galactosidase-containing liposomes are readily incorporated into the liver and spleen of mice hosting the model of globoid cell leukodystrophy, The liposome-encapsulated therapeutic enzyme, administered as a single injection after preliminary injection of liposomes with

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galactocerebroside into experimental mice, causes the breakdown of 70–80% of intracellular galactocerebroside [33].

The use of liposomes for the transfer of therapeutic enzymes through the 'blood-brain' barrier, which permits to deliver these enzymes into cells of the central nervous system also seems very attractive. It has been shown that liposome-encapsulated horseradish peroxidase acquires the ability to cross the hemato-encephalic barrier, whereas the native enzyme cannot. The presence of peroxidase in brain cells was proved by histochemical methods [34]. The same authors have shown that after injection of liposomal glucose oxidase into the rat's tail vein, up to 5% of the enzymatic activity can be discovered in brain tissues [35].

pH-sensitive liposomes for intracellular delivery

Different methods of liposomal content delivery into the cytoplasm have been elaborated [3]. According to one of these methods, the liposome is made of pH-sensitive components and, after being endocytosed in the intact form, it fuses with the endovacuolar membrane under the action of lowered pH inside the endosome and destabilizes it, releasing its content into the cytoplasm [36] (Fig. 2). Thus, endosomes become the gates from the outside into the cell cytoplasm [37]. Because this approach was reviewed many times in various publications (in 2004, the endosomal escape by pH-sensitive drug delivery systems was specifically discussed in a special issue of *Advanced Drug Delivery Reviews* #56, Leroux, J. C., ed.), here we will briefly consider only some recent examples of this approach. Cellular drug delivery mediated by pH-sensitive liposomes is not a simple intracellular leakage from the lipid vesicle because the drug has to cross also the endosomal membrane [38]. It is usually assumed that inside the endosome, the low pH and some other factors destabilize the liposomal membrane, which, in turn, interacts with the endosomal membrane provoking its secondary destabilization and drug release into the cytoplasm. The presence of fusogenic lipids in the liposome composition, such as unsaturated DOPE, with their ability to easily adopt inverted hexagonal phase, is usually required to render pH-sensitivity to liposomes [39]. Long-circulating PEGylated DOPE-containing pH-sensitive liposomes effectively deliver their contents into cytoplasm, although they have decreased pH-sensitivity [40]. Antisense oligonucleotides are delivered into cells by anionic pH-sensitive PE-containing liposomes stable in the blood, however, undergoing phase transition at acidic endosomal pH and facilitating oligo release into cell cytoplasm [41]. The study on the mechanism of endosomal delivery of genes by pH-sensitive liposomes revealed that lipids with histidine-containing head group show the involvement of imidazol ring protonation in the endosomal DNA escape [42]. New pH-sensitive liposomal additives were recently described including oleyl alcohol [43] and pH-sensitive morpholine lipids (mono-stearoyl derivatives of morpholine) [38]. Serum stable, long-circulating PEGylated pH-sensitive liposomes were also prepared using, on the same liposome, the combination of PEG and pH-sensitive terminally alkylated copolymer of N-isopropylacrylamide and methacrylic [44]. Combination of liposome pH-sensitivity and specific ligand targeting for cytosolic drug delivery utilizing decreased endosomal pH values was described for both folate and Tf-targeted liposomes [45-47]. Additional modification of pH-sensitive liposomes with an antibody results in pH-sensitive immunoliposomes. The advantages of antibody-bearing pH-sensitive liposome include cytoplasmic delivery, targetability and facilitated uptake (i.e. improved intracellular availability) via the receptor-mediated endocytosis. Successful application of pH-sensitive immunoliposomes has been demonstrated in delivery of a variety of molecules including fluorescent dyes, antitumor drugs, proteins and DNA [48].

In addition to membrane-destabilizing lipid components, there exists a large family of membrane-destabilizing anionic

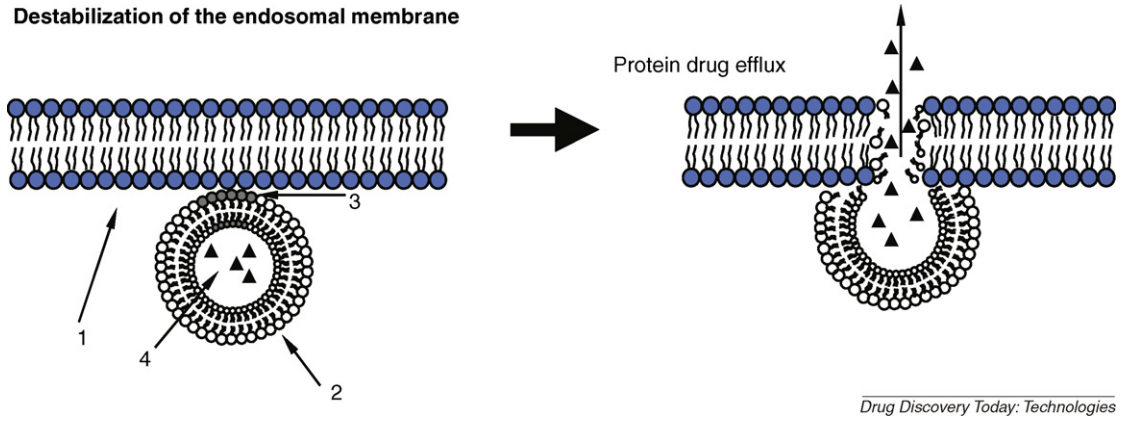

Figure 2. Schematics of endosomal drug escape with the use of pH-sensitive liposomes. 1 – endosomal membrane; 2 – endocytosed liposome; 3 – pH-sensitive liposomal component interacting with the endosomal membrane and destabilizing it; 4 – liposomal drug (modified from [18]).

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polymers that also can enhance the endosomal escape of various drugs and biomacromolecules [49]. This family includes various carboxylated polymers, copolymers of acrylic and methacrylic acids, copolymers of maleic acid, polymers and copolymers of N-isopropylacrylamide (NIPAM). Copolymers of NIPAM demonstrate lower critical solution (solubility/insolubility switch) at physiological temperatures and when precipitate, destabilize biomembranes they are interacting with [50]. Such polymers can be attached to the surface of drug/DNA-loaded liposomes or polymeric micelles allowing for endosomal destabilization and cytoplasmic escape.

Intracellular delivery of proteins by cell-penetrating peptides (CPP)

A novel approach to deliver various molecules and nanoparticles involves their modification with cell-penetrating proteins or peptides (CPPs), that is, proteins and peptides that can translocate through the cellular membranes, thereby enhancing the delivery of CPP-modified molecules inside the cell. Thus, 86-mer trans-activating transcriptional activator (TAT) from HIV-1 was efficiently taken up by various cells, when added to the surrounding media [51,52]. Subsequently, this property of translocation was found in Antennapedia (Antp), a transcription factor of *Drosophila* [53], and VP22, a herpes virus protein [54]. Their ability to translocate across the plasma membranes is confined to short sequences within these proteins of less than 20 amino acids, which are highly rich in basic residues. These peptides have been used for intracellular delivery of various cargoes with molecular weights several times greater than their own [55]. Cellular delivery using CPPs has several advantages over conventional techniques because it is efficient for a range of cell types and has a potential therapeutic application [56].

CPPs are divided into two classes: the first class consists of amphipathic helical peptides, such as transportan and model amphipathic peptide (MAP), where lysine (Lys) is the main contributor to the positive charge, whereas the second class includes arginine(Arg)-rich peptides, such as TAT (48-60) and Antp or penetratin [57].

Collectively, the recent data assume more than one mechanism for CPP-mediated intracellular delivery of various molecules and particles [58]. CPP-mediated intracellular delivery of large molecules and nanoparticles proceed via the energy-dependent macropinocytosis with subsequent enhanced escape from endosome into the cell cytoplasm [59,60], whereas individual CPPs or CPP-conjugated small molecules penetrate cells via electrostatic interactions and hydrogen bonding [61].

Because traversal through cellular membranes represents a major barrier for efficient delivery of macromolecules inside cells, CPPs might serve to ferry various molecules including proteins and peptides, into mammalian cells *in vitro* and *in vivo*. The use of peptides and protein domains with amphipathic sequences for drug and gene delivery across cellular membranes is getting increasing attention. Covalent hitching of proteins, drugs, DNA or other macromolecules onto CPPs might circumvent conventional limitations by allowing for transporting these compounds into a wide variety of cells *in vitro* and *in vivo*, as described below.

Preparations based on TAT peptide

TAT peptide (residues 1–72 or 37–72) was able to deliver heterologous proteins, such as β-galactosidase, horseradish peroxidase, RNase A and domain III of *Pseudomonas* exotoxin A into the cytoplasm of different cell types *in vitro*. TAT-β-galactosidase chimeras when tested *in vivo*, resulted in delivery to several tissues, with high levels in heart, liver and spleen, low-to-moderate levels in lung and skeletal muscle and little or no activity in kidney and brain [62]. TAT-mediated protein delivery showed potential as a therapeutic and prophylactic vaccine. Exogenous proteins cannot enter the cytosol and access the MHC class I processing pathway. So it is difficult to design a protein-based vaccine that induces class I-restricted cytotoxic T-lymphocyte (CTL) response. However, after conjugating the antigenic protein to TATp, the conjugate, such as TAT-ovalbumin conjugate, was processed by antigen presenting cells, resulting in effective killing of the target cells by antigen-specific CTLs [63,64]. TAT peptide tagged with fluorescein isothiocyanate, FITC, efficiently transduced all blood and splenic cells efficiently, together with brain and skeletal muscle cells when given intraperitoneally. Intraperitoneal injection of 116-kD β-galactosidase fused to TAT peptide, resulted in delivery of the biologically active fusion protein to all tissues in mice, including the brain [65]. The synthesis of fusion proteins requires linking the TAT transduction domain to the molecule of interest using a bacterial expression vector, followed by the purification of the fusion protein under either solubilizing or denaturing conditions. Protein transduction occurred in a concentration-dependent manner, achieving maximum intracellular concentrations in less than 10 min. Full length TAT fusion proteins ranging in size from 15 to 115 kDa demonstrated transduction in a variety of cells, such as peripheral blood lymphocytes, all blood cells, bone marrow stem cells, diploid human fibroblasts, osteoclasts, osteosarcoma, fibrosarcoma cells, glioma, heptocellular carcinoma, renal carcinoma and keratinocytes. Some of the examples of fusion proteins used for transduction *in vivo* are TAT-$p27^{Kip1}$, TAT-Cdk2dom-neg, TAT-CPP32, TAT-HSV-TK, TAT-13SE1A and TAT-pRB [66-68]. TAT PTD fused to anti-apoptotic proteins Bcl-X(L) and PEA-15 efficiently transduced pancreatic islets *ex vivo* with the prevention of apoptosis of islet cells and without affecting the insulin secretion capability of the islets. This system thus provided

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an opportunity to improve the viability of transplantable islets [69]. Fusion proteins between TAT and hemagglutinin or calcineurin A alpha stimulated osteoblast differentiation and inhibited osteoclastic resorption, providing a basis for the development of such fusion proteins using molecules involved in bone cell differentiation and function [70].

TAT fusion protein displayed potential to treat disorders pertaining to oxidative stress. TAT gene, encoding 9-mer TAT transduction domain, when fused to a human Cu, Zn-superoxide dismutase (Cu,Zn-SOD) gene produced a genetic in-frame TAT-SOD fusion protein, which transduced into the cells in time-dependent and dose-dependent fashion, much more efficiently than the native proteins, opening the doors to replenish the Cu,Zn-SOD in various disorders related to this antioxidant enzyme [71]. Similarly, a human liver catalase (CAT) gene when fused with a gene encoding for 9-mer TAT peptide and arginine-rich peptides to produce genetic in-frame TAT-CAT and 9Arg-CAT fusion proteins, respectively, transduced mammalian cells in a time-dependent and dose-dependent fashion. The viability of transduced cells when exposed to oxidative stress increased significantly. Further, TAT-CAT and 9Arg-CAT fusion proteins efficiently penetrated the epidermis as well as the dermis of the subcutaneous layer when sprayed on animal skin, suggesting the use of such fusion proteins for various disorders requiring the application of antioxidant enzymes [72].

TAT fusion proteins could also deliver biologically active exogenous heat shock protein 70 (HSP70), required for the cytoprotection against cellular stressors [73], and human glutamate dehydrogenase (GDH) in case of GDH-deficient disorders [74]. TAT was also used to transduce a biologically active neuroprotectant Bcl-xL in cerebral ischemia. TAT and Bcl-xL fusion protein resulted in robust protein transduction in cultures and also delivered the protein across the blood-brain barrier. When given intraperitoneally, the fusion protein transduced brain cells within 1–2 h and decreased cerebral infarction in a dose-dependent manner after 90 min of focal ischemia. When administered i.p. in gerbils, it reduced ischemic injury of hippocampal CA1 neurons. The fusion protein was effective when given even after ischemia (up to 45 min) [75].

TAT transduction domain also showed a potential for application in inflammatory conditions. The transcription factor NF-κB is a mediator of inflammation. Proinflammatory cytokines such as TNF-α and IL-1β mediate their action by activating NF-κB; therefore, methods to reduce NF-κB activity will be beneficial in chronic inflammatory conditions. The nonphosphorylatable, nondegradable superrepressor IκBα (srIκBα) mutant strongly inhibits NF-κB activity. The fusion of this mutant with TAT peptide resulted in rapid and efficient uptake in the cells, inhibition of TNF-α or IL-1β-induced NF-κB activation in a dose-dependent manner and NF-κB-mediated transcription. This approach thus provided a novel way to regulate inflammation [76].

Other cell-penetrating peptides

Herpes Simplex Virus VP22 protein was used to deliver E2 protein to target cells. Overexpression of the E2 protein in cervical cancer cells can induce growth arrest and/or apoptotic cell death, thus E2 might be useful in the treatment of cervical cancer. VP22-E2 fusion proteins induced apoptosis in transiently transfected human papillomavirus (HPV)-transformed cervical carcinoma cell lines. When COS-7 cells producing VP22-E2 were seeded into cultures of HPV-transformed cells, VP22-E2 entered the non-producing cells and induced apoptosis suggesting that the local delivery of VP22-E2 fusion proteins could be used to treat cervical cancer and other HPV-associated diseases [77]. A chimeric VP22-SV40 large T antigen fusion protein spread into terminally differentiated myotubes where it accumulated in the nucleus. This fusion protein retained the ability to override the cell cycle arrest as shown for SV40 large T antigen alone. The transduced fusion protein remained capable of inducing S-phase and mitosis in these otherwise terminally differentiated cells, opening the way to exploit this novel strategy for tissue regeneration [78]. VP22 enhanced intercellular trafficking of thymidine kinase (TK) and amplified the TK/ganciclovir (GCV) killing effect, especially in the lower range of GCV concentrations, offering a new strategy to enhance the effectiveness of suicide gene therapy for the treatment of cancers [79].

Chimeric polypeptides of VP22 linked to the entire p53 protein, were shown to spread between cells and accumulate in recipient cell nuclei. Also the VP22-p53 chimeric protein efficiently induced apoptosis in p53 negative human osteosarcoma cells resulting in widespread cytotoxic effect [80].

Transportan and Antp were used for the intracellular delivery of peptide nucleic acids (PNA) [81]. Recently, a membrane-translocating motif composed of 12 amino acids was attached to the recombinant mouse CP-SOCS3 protein (suppressor of cytokine signaling) and intracellular therapy with such conjugate inhibited inflammation and apoptosis in mice [82].

Intracellular delivery of therapeutics antibodies by cell-penetrating peptides

In certain cases, the antibodies need to be delivered intracellularly for their tumoricidal effect [83]; however, immunoglobulins by themselves cannot cross the plasma membrane. Different methods used for the intracellular delivery of antibodies, such as electroporation or microinjection, have clear limitations because these methods result in disruption of cellular membranes and decreased cell viability [14,15]. Therefore, CPPs were considered for the delivery of antibodies inside cells.

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Table 1. Methods to deliver protein and peptide drugs inside cells

Method	Advantages	Limitations	Applicability
Traditional methods, such as electroporation or microinjection	Control on the level of the individual cell	Invasive, cannot be used in clinical conditions	*In vitro* experiments
pH-sensitive drug delivery systems	Non-invasive, can be used for systemic applications	Non-specific potential problems, with scaling up	*In vitro*, preclinical *in vivo* experiments
Use of cell-penetrating peptides	Non-invasive, can be used for systemic applications	Low specificity, cost	*In vitro*, preclinical *in vivo* experiments

Peptide analogs of the 37–62-sequence region of the TAT protein when conjugated to poorly internalizable antitumor antibody Fab fragments, enhanced the *in vitro* cell surface retention and internalization of these fragments to the level of the whole antibodies [84].

TAT peptide (37–72) was used to neutralize tetanus toxin (TET) inside cells. Because of the very slow degradation of TET in nerve cells, anti-TET antibodies are required for the neutralization of TET. The conventional method of electroporation for the delivery of antibodies cannot be used in a clinical situation. However, conjugates between anti-tetanus $F(ab')_2$ fragments and TAT peptide (37–72) could be taken up by cells, the disulfide conjugate being the one that could neutralize tetanus toxin inside the cells [84].

Another novel approach to deliver antibodies includes the use of TAT-fused protein. Genetically engineered fusion protein consisted of two functional domains, the TAT PTD and the B domain of staphylococcal protein A (SpA), which binds to the Fc fragment of IgG. TAT-SpA fusion protein when conjugated to IgG, showed the intracellular delivery of antibody in a time and dose-dependent manner [85].

Despite all these studies, interestingly enough, when the biodistribution studies on TAT peptides (44–57)-antibody conjugates were prepared, conjugated antibody showed severely reduced tumor targeting activity compared with unmodified antibody, thereby raising certain concern about the use of CPPs for the delivery of antibodies *in vivo* [86].

Theoretically, the whole variety of delivery functions can be assembled on a single protein-loaded pharmaceutical carrier as shown in Fig. 3 for liposomes. Here, we can see simultaneously sterically protecting polymer providing longevity, antibodies allowing for the specific targeting and cell-penetrating functions delivering protein and peptide drugs inside cells.

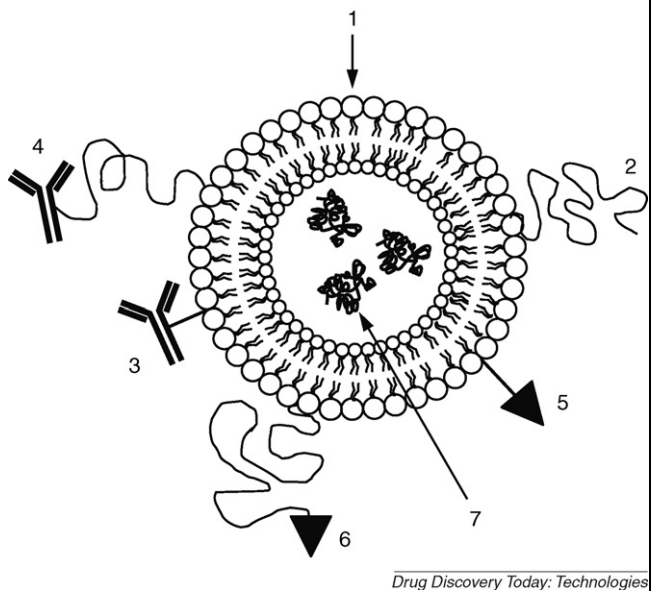

Figure 3. Liposome as a multifunctional carrier for protein drug delivery. 1 – lipid bilayer; 2 – protective polymer; 3 – vector molecule attached to the liposome surface; 4 – vector molecule attached to the liposome via the polymeric chain; 5 – protein transduction domain; 6 – cell-penetrating peptide attached to the liposome via the polymeric chain; 7 – protein drug.

Conclusion

Thus, our current knowledge provides some promising approaches on how to deliver peptide and/or protein-based drugs not only to the site of disease but also inside target cell for enhanced therapy. Traditional methods of intracellular delivery, such as electroporation or microinjection are invasive and applicable for *in vitro* experiments, but not for clinical conditions. The use of various pharmaceutical nanocarriers, such as liposomes, possessing pH-sensitivity and being able to escape from the endosomes upon the endocytic uptake, or the modification of peptide and protein drugs with cell-penetrating peptides, can allow for efficient and non-invasive intracellular delivery. Although the majority of experiments with pH-sensitive pharmaceutical nanocarriers and cell-penetrating peptide-modified drugs and drug carriers are still on pre-clinical stage, we can expect the appearance of new drugs and treatment protocols based on these methods in the very near future (Table 1).

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